

Downregulation of nuclear and cytoplasmic Chibby is associated with advanced cervical cancer

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Abstract. Chibby has been identified as a putative tumor suppressor and antagonist to β -catenin, thereby controlling the Wnt signaling pathway. Chibby is typically downregulated in numerous types of cancer and may be associated with tumorigenesis. The present study aimed at clarifying the following: i) Whether Chibby antagonizes β -catenin in cervical cancer; ii) whether Chibby and β -catenin mRNA expression is associated with cancer progression; and iii) whether Chibby and β -catenin expression may be used as a biomarker. A total of 87 paraffin-embedded cervical sections with distinct cervical intraepithelial neoplasia (CIN) stages (chronic cervicitis, CIN 1, CIN 2, CIN 3 and invasive squamous cell carcinoma) were collected between June 2004 and October 2012. The mRNA expression level of Chibby and β -catenin was determined using the polymerase chain reaction. Protein expression and cellular localization of Chibby and β -catenin were determined using immunohistochemistry. Chibby and β -catenin were analyzed for possible association with the progression of cervical cancer. Chibby mRNA expression and the Chibby/ β -catenin ratio were identified to be downregulated in invasive tumors. Positive cytoplasmic and nuclear staining for Chibby was associated with CIN staging and decreased as the CIN stage increased. In addition, the cytoplasmic and membrane intensity of β -catenin was associated with invasive

tumors, in which a significantly increased level of protein expression was detected. Chibby may be a tumor suppressor in cervical cancer, since the dysregulation of Chibby expression is associated with tumorigenesis in cervical cancer. Chibby and β -catenin expression together may potentially to a biomarker for disease progression in cervical cancer.

Introduction

Cervical cancer is the most prevalent type of cancer in females (1) with >500,000 novel cases reported annually in developing countries (2), and >200,000 cervical cancer-associated mortalities each year worldwide (3). Signaling pathway dysregulation is hypothesized to be involved in cervical cancer tumorigenesis (4). The highly conserved Wnt signaling pathways, are involved in cell differentiation, embryonic development and tissue formation. The canonical Wnt signaling pathway is also known as the β -catenin pathway and is responsible for triggering transcription of a subset of downstream genes. The Wnt signaling pathway serves a crucial function in development and the dysregulation of this pathway is associated with a number of disorders, including cancer. The principal reasons for Wnt signaling dysfunction are mutation or malfunction of proteins involved in the pathway (5). Abnormal accumulation of β -catenin is suggested to stimulate the proliferation and metastasis of cancer cells (6-8).

β -catenin is an 88 kDa multi-function scaffolding protein encoded at chromosome 3p22-21.3 and is primarily membrane bound with epithelial (E)-cadherin. β -catenin accounts for the following two biological functions: i) Acts as a subunit of the adhering complex when binding with E-cadherin and α -catenin; and ii) acts as a co-activator of the transcription factor (TCF)/lymphoid enhancer-binding factor family (LEF) (9,10). Cytoplasmic and nuclear accumulation of β -catenin are a feature of colorectal, lung and breast cancer, hepatoma and melanoma (11-13). Additionally, β -catenin has been identified to be overexpressed in cervical cancer (8) and the dysregulation of β -catenin has been suggested to be a biomarker of cervical squamous cell carcinoma (14). A number

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of underlying molecular mechanisms have been suggested to explain how the abnormal accumulation of β -catenin leads to cancer vulnerability, including interference with cellular polarity and accelerated cell proliferation, survival and migration (5,15).

Chibby is a highly conserved protein with a molecular weight of 14.5 kDa comprising 126 amino acids and encoded at chromosome 22q12-13. Chibby has been identified to be an antagonist of β -catenin; it binds to β -catenin in the nucleus, and exhibits a negative regulating effect on the Wnt signaling pathway and the transcriptional activities of genes downstream of Wnt (16). In addition, Chibby has been hypothesized to be a tumor suppressor that controls β -catenin through two primary mechanisms, including a direct control, nuclear-cytoplasmic shuttling of Chibby controls β -catenin signaling and indirect control, which cooperates with 14-3-3 in order to regulate β -catenin subcellular distribution and signaling activity (17,18). Chibby is suggested to be downregulated in neuroblastoma (19), lung cancer (20), ependymoma (21) and colorectal cancer (22,23). Furthermore, mutations of Chibby have been associated with tumorigenesis (24). However, the association between Chibby and β -catenin, and the underlying molecular mechanism by which Chibby may suppress cervical cancer tumorigenesis, remains unknown. From preliminary data, overexpression of Chibby may suppress the transcriptional activity of β -catenin and induce apoptosis. Consequently, cell proliferation, migration and cancer cell colonization were inhibited, which indicated a tumor suppressor function of Chibby (Huang, Y.L., unpublished work). Therefore, we hypothesized that Chibby may control cancer cell growth by negatively regulating β -catenin/TCF4 signaling and affecting c-Myc and proliferating cell nuclear antigen expression. In two cervical cancer cell lines, HeLa and SiHa, mRNA and protein Chibby expression were downregulated. These results suggested that Chibby may be a tumor suppressor in cervical cancer *in vitro* (Huang, Y.L., unpublished work); however, the precise *in vivo* function of Chibby in cervical cancer, the differences between Chibby expression in carcinoma and benign tissue, and the association between Chibby and β -catenin expression all remain unknown. Therefore, in the present study, paraffin-embedded cervical cancer tissues at distinct cervical intraepithelial neoplasia (CIN) stages were selected and the mRNA expression of Chibby and β -catenin was quantified. In addition, protein expression and localization were determined to explore: i) The cytosolic and nuclear expression patterns with respect to Chibby and β -catenin; ii) whether mRNA expression of Chibby and β -catenin is associated with the progression of cervical cancer; and iii) whether Chibby and β -catenin expression may be used as a biomarker of cervical cancer.

Materials and methods

Tissue collection. All paraffin-embedded tissues were retrospectively collected from the tissue bank of Kaohsiung Armed Forces General Hospital (KAFGH; Kaohsiung, Taiwan) between June 2004 and October 2012. Patient's age ranged from 19 to 89 years with an average of 49.09 years. A total of 87 cervical tissues were selected and dissected from cervical conization and total hysterectomy, and no biopsy samples were used. All participants were females. Tissues selected were 4

chronic cervicitis, 13 CIN 1, 15 CIN 2, 33 CIN 3 and 22 invasive squamous-cell carcinoma (SCC) samples, based on the classification system described previously (25). The present study was approved by the Institutional Review Board (IRB) of KAFGH (Kaohsiung, Taiwan).

Immunohistochemistry of β -catenin and Chibby. The paraffin-embedded tissue blocks were pre-sliced using a tissue dissector (thickness, 1-2 μ m). Subsequently, paraffin blocks were cooled in an ice-water mixture for 30 min prior to sectioning. Tissue sections 4-6 μ m thick were cut and placed on histone-coated (Hate Chemical, Westbury, NY, USA) slides. Following a brief drying period of approximately 15 min, the tissue sections were heat-fixed to the slide at 37°C and subsequently incubated at 70°C for 30 min before deparaffinization. The slides were then taken directly from the incubator and deparaffinized in xylene twice for 5 min each, followed by one rinse in absolute ethanol (ETOH), and then sequentially rinsed in 90, 80 and 70% ETOH and dH₂O for 10 sec each. To eliminate staining due to endogenous peroxidase, the tissue sections were treated with an ETOH/H₂O₂ (45 ml ETOH/3 ml H₂O₂) block for 10 min at 42°C (26). Immunohistological staining was performed using a NovoLink polymer detection kit (Leica Microsystems, Ltd., Milton Keynes, UK). A mouse monoclonal immunoglobulin G (IgG) antibody (Ab) was used to detect β -catenin (monoclonal mouse anti- β -catenin Ab; catalog no. NCL-B-CAT; dilution, 1:200; Leica Microsystems, Ltd.) and a rabbit polyclonal IgG Ab (polyclonal rabbit anti-Chibby Ab; catalog no. HRIHFB2025; dilution, 1:400; ABgene; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to detect Chibby. Slides were incubated for 30 min at 42°C with the primary antibodies. Slides were then rinsed in PBS and subsequently incubated in the presence of the secondary antibody at 42°C for 15 min. The secondary antibodies were included in the NovoLink polymer detection kit. All procedures were performed according to the manufacturer's protocol. The results were observed under a Nikon Eclipse 50i light microscope (Nikon, Tokyo, Japan) at magnifications of x40-400 and were interpreted by senior pathologists who were blinded to the diagnostic results of each section. Overall, 90% of the samples were consistent with their original diagnosis; if any uncertainties were encountered, the section was re-interpreted by a different pathologist. The protein expression level was interpreted by two means, staining intensity and the positive staining area; in addition, the protein location corresponding to cytosol and nuclear was indicated. For determining intensity, the following four-level scale was used: No (no protein positive staining can be observed at x400 magnification), weak (protein positive staining can easily be observed at x400 magnification), intermediate (protein positive staining can easily be observed at x200 magnification) and strong staining (protein positive staining can easily be observed at x100 magnification). For defining the area positive staining, the following five-level scale was used: No staining, <1%, between 1 and 10%, between 11 and 50%, and >50% positivity. For those sections with positive detecting area <10% was considered as 'negative' for protein expression; in contrast, sections with >10% positive staining area were viewed as 'positive' for target protein expression.

Table I. mRNA expression with respect to β -catenin and chibby in different CIN stages of cervical cancer.

Variable	CIN	N	Mean rank	χ^2	Df	Asymp. sig. ^{a,b}
Chibby mRNA expression	CIN 1	11	39.95	15.57	3	0.001
	CIN 2	9	22.22			
	CIN 3	25	29.06			
	Invasive tumor	9	13.22			
	Total	54				
β -catenin mRNA expression	CIN 1	11	34.77	3.95	3	0.267
	CIN 2	9	22.89			
	CIN 3	25	25.06			
	Invasive tumor	9	30.00			
	Total	54				
Chibby/ β -catenin ratio	CIN 1	8	30.09	10.07	3	0.018
	CIN 2	9	28.78			
	CIN 3	25	31.32			
	Invasive tumor	9	12.44			
	Total	51				

^aKruskal Wallis test; ^bgrouping variable; CIN, cervical intra-epithelial neoplasia; Df, degree of freedom; Asymp. sig. asymptotic significance; Chibby, an antagonist of β -catenin.

RNA extraction. Total RNA was prepared from tissue sections prepared from paraffin-embedded tissues using a PureLink FFPE Total RNA Isolation kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and de-paraffinization, purification and washing were conducted according to the manufacturer's protocol. The RNA concentration was determined by detecting the absorbance at 280 nm under ultraviolet.

Complementary (c)DNA synthesis. First strand cDNA synthesis was carried out using an Impron-II Reverse Transcription System (Promega Corporation, Madison, WI, USA). Total RNA (1 μ g) was premixed with oligo(dT) and random hexamers in a 0.2 ml vial, heated at 70°C for 5 min and chilled at 4°C for 5 min for pre-denaturation. Subsequently, 4 μ l Impron-II 5x Reaction Buffer, 25 mM MgCl₂, 1 μ l 10 mM dNTP Mix, 20 U ribonuclease inhibitor, nuclease-free water and 1 μ l ImProm-II Reverse Transcriptase were added to a final volume of 15 μ l. The following temperature protocol was applied: 25°C for 5 min for primer annealing, 42°C for 60 min for synthesis, followed by 70°C for 15 min to inactivate enzymes. The synthesized cDNA was stored at -20°C until used.

Quantitative polymerase chain reaction (qPCR). A total of 11 CIN 1, 9 CIN 2, 25 CIN 3 and 9 SCC cancerous tissues underwent target mRNA detection (only 54 out of 83 collected cervical tumor tissues had sufficient quality mRNA for further tests). Detection of mRNA expression levels with respect to endogenous Chibby and β -catenin was performed using EZtime real-time PCR premix (2X SYBR Green premix, Yeastern Biotech Corp., Taipei, Taiwan). The thermal cycling protocol was performed using an IQ5 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The increase in fluorescence emission (Rn) was measured during PCR amplification, and the difference (Δ Rn) between the fluorescence

emission of the product and the baseline was calculated using IQ5 Optical System Software version 2.1 (Bio-Rad Laboratories, Inc.) and plotted against the cycle number. Cycle threshold (CT) values were calculated by determining the number of thermal cycles at which the emitted fluorescence exceeded the threshold point, as described previously (27). The reaction mixture contained 10 ng cDNA diluted in 2.5 μ l diethylpyrocarbonate-treated water (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 12.5 μ l (2X) EZtime SYBR Green PCR premix (Yeastern Biotech Corp.) and 2 μ l gene-specific primers (final concentration, 50 nM each) in a final reaction volume of 25 μ l. The reaction conditions for Chibby and β -catenin were as follows: 95°C for 10 min (denaturation of the template and activation of DNA polymerase), followed by 45 cycles of 95°C for 20 sec (denaturation of PCR products), 58°C for 1 min (primer annealing), and 72°C for 15 sec (extension). The reaction conditions for β -actin were as follows: 95°C for 10 min (denaturation of the template and activation of DNA polymerase), followed by 45 cycles of 95°C for 10 sec (denaturation of the PCR products), 58°C for 1 min (primer annealing), and 55°C for 15 sec (extension). Each real-time PCR was performed in duplicate to evaluate data reproducibility. A melting curve analysis of the PCR products was generated following amplification by heating the reaction mixtures between 60°C and 95°C with a rate of 0.1°C/sec, and continuously acquiring fluorescence emission data. The melting temperatures (T_m) of the target genes and β -actin amplicons were expected to be ~80.0°C and 85.0°C, respectively, whereas primer-dimers and/or other non-specific products were characterized by a decreased T_m (\leq 75.0°C). Calculations and validation of the comparative CT (2^{- $\Delta\Delta$ C_q}) method (normalizing to an endogenous reference provides a method for correcting results for differing amounts of input RNA) were used for target gene mRNA quantification (28). β -actin was used as a reference

Table II. mRNA expression level with respect to β -catenin and chibby in various CIN stages of cervical cancer.

Variable	CIN 1, n (%)	CIN 2, n (%)	CIN 3, n (%)	Invasive, n (%) tumor	Median	χ^2	Df	Asymp. sig.
Chibby mRNA expression	>Median	9 (81.82)	4 (44.44)	1 (11.11)	486.62	10.05	3	0.018
	\leq Median	2 (18.18)	5 (55.56)	8 (88.89)				
β -catenin mRNA expression	>Median	8 (72.73)	4(44.44)	4 (44.44)	663.47	2.86	3	0.415
	\leq Median	3 (27.27)	5 (55.56)	5 (55.56)				
Chibby/ β -catenin ratio	>Median	7 (63.64)	6 (66.67)	0 (0.0)	1.91	11.18	3	0.011
	\leq Median	4 (36.36)	3 (33.33)	9 (100)				

CIN, cervical intra-epithelial neoplasia; Df, degree of freedom; Asymp. sig., asymptotic significance; Chibby, an antagonist of β -catenin; >Median, the mRNA expression is greater than the median value; \leq Median, mRNA expression is equal to or less than median value.

gene to normalize all PCRs for the amount of loaded RNA. All primer pairs were designed using a web-based program 'GenScript Real-time PCR (TaqMan) Primer Design' provided by GeneScript.com. The sequences of primer pairs were as follows: Chibby forward (F), 5'-TCTGGGCTACAGAGTCCTTG-3'; Chibby reverse (R), 5'-TGTCTTCTTCGGACTGAACG-3'; β -catenin F, 5'-GCAATCCCTGAACTGACAAA-3'; β -catenin R, 5'-TGAGGAGAACGCATGATAGC-3'; β -actin F, 5'-GACATCCGCAAAGACCTGTA-3'; β -actin R, 5'-GGAGCAATGATCTTGATCTTCA-3'.

Statistical analysis. All statistical procedures were conducted using SPSS software (version 22.0; IBM Corp, Armonk, NY, USA). The expression levels of target mRNAs were analyzed using non-parametric statistical methods, including the Kruskal-Wallis test and median test, to discriminate whether inter-group differences were significant among the CIN staging groups (including chronic cervicitis, CIN 1, CIN 2, CIN 3 and invasive SCC). For Kruskal-Wallis test, all the mRNA expression levels for the four CIN groups were entered into one column and rank ordered from lowest to highest. Once the ranks were assigned, the scores were split back into the four groups. Subsequently, the mean of the *ranks* in each group were computed, and Kruskal-Wallis test was performed to determine whether there was a statistically significant difference in the mean ranks for each group. The χ^2 test was used to determine whether protein staining intensity and positive staining area were significantly different among different CIN groups. The statistical results were presented separately by the different subcellular locations. In addition, Chibby/ β -catenin ratio was evaluated and compared for any difference among CIN groups. Finally, logistic regression was applied to distinguish factors associated with CIN stage and/or invasive tumor. In addition, the data was categorized as a dummy variable (29) according to the criteria 01, by which samples with a Chibby nucleus and cytoplasmic positive staining area <1% and a β -catenin cytoplasmic intensity that was greater than or equal to intermediate. Membrane intensity was identified as strong staining and was indicated as 1; whereas the counterparts were indicated as 0. The aforementioned dummy variable of criteria 01 was analyzed using binary logistic regression to determine whether it was associated with SCC. The positive prediction value (PPV; the probability that the criteria 01-positive tumors were SCC), the negative prediction value (NPV; the probability that criteria 01-negative tumors were non-SCC) and accuracy (refers to the probability that true SCC and non-SCC samples can be predicted by criteria 01 from overall samples) were used as performance measures. P<0.05 was considered to indicate a statistically significant difference.

Results

Chibby mRNA expression is downregulated in SCC, but not β -catenin. A total of 54/83 paraffin embedded cervical cancerous tissues were subjected to detection of Chibby and β -catenin mRNA expression due to a relatively good RNA preparation. PCR efficiency with respect to β -catenin and Chibby amplification ranged between 90 and 110%, and the standard curves of the two genes were obtained with a slope of -3.3 and an R squared >0.99 (the coefficient value of a standard curve), which

Table III. Protein expression with respect to β -catenin and chibby in different CIN stages of cervical cancer.

Staining	Count, n (%)						χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor				
None	0 (0.0)	0 (0.0)	1 (6.7)	4 (12.1)	11 (50.0)		29.258	12	0.004
Weak	3 (75.0)	7 (53.8)	11 (73.3)	18 (54.5)	10 (45.5)				
Intermediate	1 (25.0)	5 (38.5)	3 (20.0)	11 (33.3)	1 (4.5)				
Strong	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)				
Total count, n	4	13	15	33	22				
B, Chibby nucleus area									
Staining	Count, n (%)						χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor				
None	0 (0.0)	0 (0.0)	1 (6.7)	4 (12.1)	11 (50.0)		60.179	16	<0.001
<1	3 (75.0)	1 (7.7)	0 (0.0)	2 (6.1)	1 (4.5)				
1-10	1 (25.0)	0 (0.0)	2 (13.3)	0 (0.0)	5 (22.7)				
11-50	0 (0.0)	2 (15.4)	3 (20.0)	8 (24.2)	0 (0.0)				
>50	0 (0.0)	10 (76.9)	9 (60.0)	19 (57.6)	5 (22.7)				
Total count, n	4	13	15	33	22				
C, Chibby cytoplasm intensity									
Staining	Count, n (%)						χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor				
None	2 (50.0)	0 (0.0)	11 (73.3)	25 (75.8)	20 (90.9)		49.101	12	<0.001
Weak	2 (50.0)	4 (30.8)	1 (6.7)	6 (18.2)	2 (9.1)				
Intermediate	0 (0.0)	7 (53.8)	2 (13.3)	1 (3.0)	0 (0.0)				
Strong	0 (0.0)	2 (15.4)	1 (6.7)	1 (3.0)	0 (0.0)				
Total count, n	4	13	15	33	22				

Table III. Continued.

Staining	Count, n (%)							χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor					
None	0 (0.0)	0 (0.0)	1 (6.7)	4 (12.1)	11 (50.0)			29.258	12	0.004
None	2 (50.0)	0 (0.0)	11 (73.3)	25 (75.8)	20 (90.9)			60.179	16	<0.001
<1	1 (25.0)	2 (15.4)	0 (0.0)	0 (0.0)	1 (4.5)					
1-10	1 (25.0)	2 (15.4)	0 (0.0)	0 (0.0)	1 (4.5)					
11-50	0 (0.0)	9 (69.2)	4 (26.7)	6 (18.2)	0 (0.0)					
>50	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.1)	0 (0.0)					
Total count, n	4	13	15	33	22					
E, β -catenin nucleus intensity										
Staining	Count, n (%)							χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor					
ND	0 (0.0)	0 (0.0)	2 (13.3)	0 (0.0)	3 (13.6)					ND
None	4 (100.0)	13 (100.0)	13 (86.7)	33 (100.0)	19 (86.4)					
Total count, n	4	13	15	33	22					
F, β -catenin nucleus area										
Staining	Count, n (%)							χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor					
ND	0 (0.0)	0 (0.0)	2 (13.3)	0 (0.0)	3 (13.6)					ND
None	4 (100.0)	13 (100.0)	13 (86.7)	33 (100.0)	19 (86.4)					
Total count, n	4	13	15	33	22					

Table III. Continued.

Staining	Count, n (%)						χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor				
ND	0 (0.0)	0 (0.0)	2 (13.3)	0 (0.0)	3 (13.6)			ND	
Weak	0 (0.0)	12 (92.3)	5 (38.5)	15 (45.5)	5 (26.3)				
Intermediate	0 (0.0)	1 (7.7)	4 (30.8)	8 (24.2)	7 (36.8)				
Strong	0 (0.0)	0 (0.0)	4 (30.8)	10 (30.3)	7 (36.8)				
Total count, n	4	13	13	33	19				
H, β -catenin cytoplasm area									
Staining	Count, n (%)						χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor				
None	4 (100.0)	0 (0.0)	2 (13.3)	0 (0.0)	3 (13.6)		71.498	<0.001	
<1	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)	0 (0.0)				
1-10	0 (0.0)	1 (7.7)	1 (6.7)	0 (0.0)	0 (0.0)				
11-50	0 (0.0)	12 (92.3)	3 (20.0)	5 (15.2)	2 (9.1)				
>50	0 (0.0)	0 (0.0)	9 (60.0)	27 (81.8)	17 (77.3)				
Total count, n	4	13	15	33	22				
I, β -catenin membrane intensity									
Staining	Count, n (%)						χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor				
None	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)		7.831	0.251	
Weak		1 (7.7)	2 (15.4)	8 (24.2)	2 (10.5)				
Intermediate		6 (46.2)	4 (30.8)	12 (36.4)	3 (15.8)				
Strong		6 (46.2)	7 (53.8)	13 (39.4)	14 (73.7)				
Total count, n	4	13	13	33	19				

Table III. Continued.

Staining	Count, n (%)						χ^2	df	P-value
	Chronic cervicitis	CIN 1	CIN 2	CIN 3	Invasive tumor				
<1	4 (100.0)	0 (0.0)	2 (13.3)	0 (0.0)	3 (13.6)	33.206	12.0	0.001	
1-10	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)	0 (0.0)				
11-50	0 (0.0)	2 (15.4)	2 (13.3)	1 (3.0)	2 (9.1)				
>50	0 (0.0)	11 (84.6)	11 (73.3)	31 (93.9)	17 (77.3)				
Total count, n	4	13	15	33	22				

CIN, cervical intra-epithelial neoplasia; df, degree of freedom. ND, not done.

revealed successful amplification (Fig. 1). A non-parametrical approach was adopted for analyzing mRNA expression, since the expression data was unlikely to be normally distributed. As presented in the Table I, using the Kruskal-Wallis test, the raw data of mRNA expression was converted into ranking values. Chibby mRNA (P=0.001) and the Chibby/ β -catenin ratio (P=0.018), but not β -catenin (P=0.267), differed among the CIN groups. Progressively decreased expression levels of Chibby (mean rank, 13.22) and decreased Chibby/ β -catenin ratio (mean rank, 12.44) were identified when compared with CIN 1 to CIN 3 tumors (>20 mean rank). Using the median test, Chibby mRNA (Table II, line 2, 8/9 tumors; 88.89%) and Chibby/ β -catenin ratio (Table II, line 6, 9/9 tumors; 100%) revealed an increased proportion in the mRNA expression level below the median value in invasive tumors compared with CIN 1, 2 and 3 tumors, which suggested that Chibby mRNA was downregulated significantly in SCC (Chibby mRNA, P=0.018; Chibby/ β -catenin ratio, P=0.011; Table II).

Chibby nucleus and cytoplasmic staining are associated with CIN stage. Chibby was determined in all chronic cervicitis samples with a weak to intermediate intensity and <10% positivity area in the nucleus. In addition, Chibby was identified with weak intensity and <10% positivity area in the cytoplasm in half of the chronic cervicitis tissues (Table III; Fig. 2A and B). In the nucleus and cytoplasm, the intensity of Chibby was identified to be decreased in invasive tumor (45.5% weak positivity in nucleus; 9.1% weak positivity in the cytoplasm) compared with the other groups (Table III, part A, nucleus, P=0.004; part C, cytoplasm, P<0.001). Notably, the positive staining area of Chibby in the nucleus (>50% positivity; Table III) and cytoplasm (11-50% positivity; Table III) decreased as the CIN stage increased (Table III; P<0.001; Fig. 2C-J).

β -catenin cytoplasmic and membrane staining intensity are associated with CIN stage. β -catenin protein expression was detected in the cytoplasm and membrane, but not in the nucleus (Table III, part E-J). Specifically, β -catenin was frequently detected at the sites where proximal to cell membrane (Fig. 3). In chronic cervicitis tissues, no β -catenin expression was detected in any of the samples. Cytoplasmic β -catenin expression was increased in CIN 1 (weak intensity, 11-50% positive staining area, 12/13, 92.3%) and CIN 3 (>50% positive staining area, 27/33, 81.8%) stage tumor compared with the invasive tumor, with the intensity principally ranging between intermediate and strong (14/19; 73.7%) and a positive staining area >50% (17/22; 77.3%) in invasive tumors (Table III, part G&H; P<0.001). An identical pattern was observed in membrane β -catenin, where the intensity was primarily identified as strong (14/19; 73.7%) and a positive staining area of >50% (17/22; 77.3%) in the invasive tumor group. However, no inter-group differences were identified in β -catenin membrane intensity between the different CIN staging groups (Table III, part I, P=0.251). The results of the present study may suggest that β -catenin is overexpressed in the cytoplasm and membrane in advanced CIN tumors, and may be an early event associated with cervical cancer tumorigenesis. To clarify whether and which factor was associated with CIN stage and invasive tumors, multi-nominal logistic regression was performed, and the results are presented in

Table IV. Multi-nominal regression analysis to predict factors associated with CIN staging.

Effect ^a	Model fitting criteria		Likelihood ratio tests		
	-2 Log likelihood of reduced model	χ^2	df	P-value	
Intercept	40.109	0.000	0	.	
Chibby nucleus area	72.127	32.017	12	0.001	
Chibby cytoplasmic intensity	52.130	12.021	6	0.062	
Chibby cytoplasmic area	73.933	33.824	9	<0.001	
β -catenin cytoplasmic intensity	58.017	17.908	6	0.006	
β -catenin membrane intensity	55.452	15.343	6	0.018	

CIN, cervical intra-epithelial neoplasia; ^adependent, CIN staging.

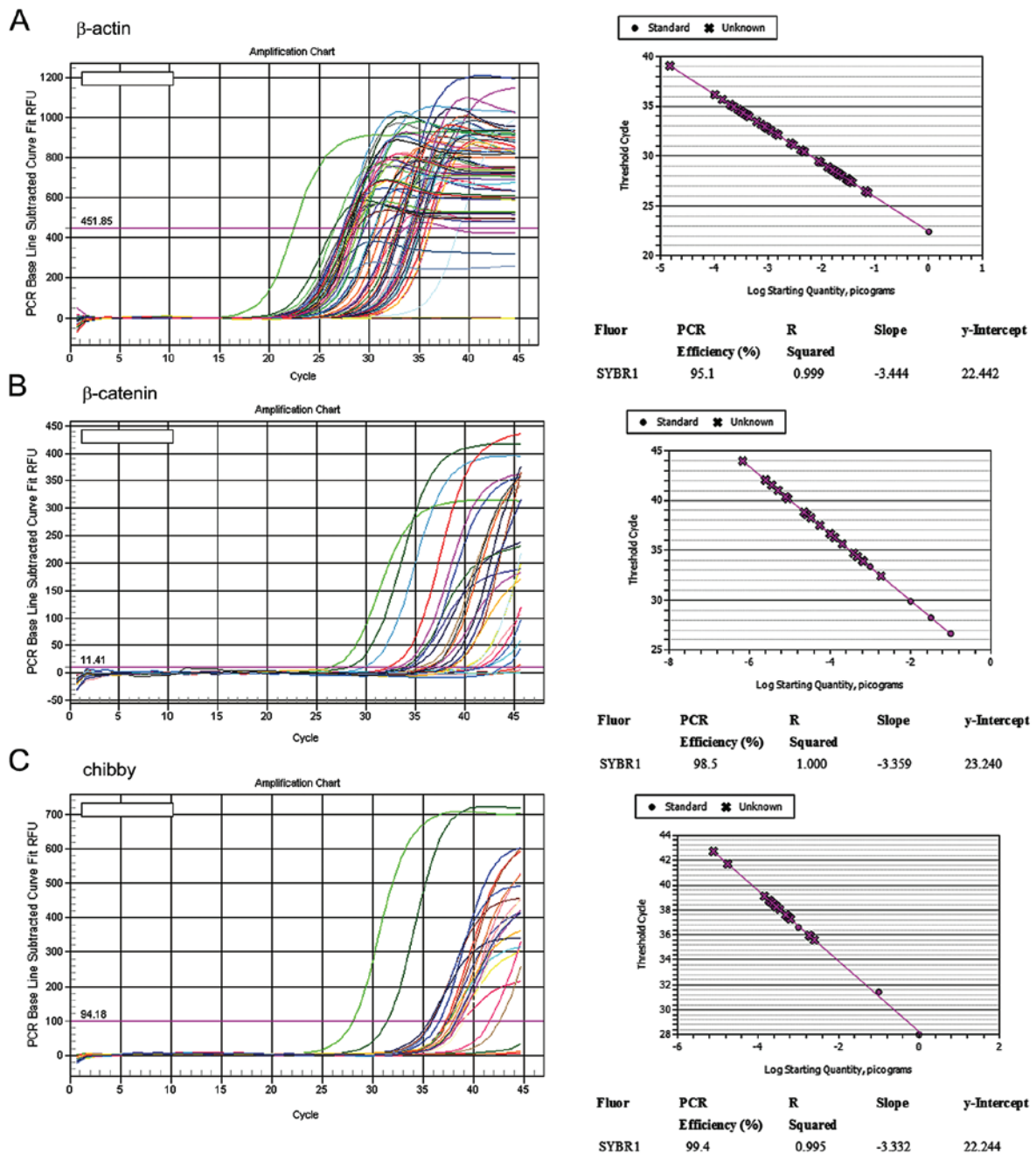


Figure 1. Amplification plots (left) and standard curves (right) of quantitative PCR. (A) β -actin; (B) β -catenin; and (C) Chibby. PCR, polymerase chain reaction; Fluor, fluorophore; RFU, relative fluorescence units.

Table V. Results of logistical regression that was aimed at determining whether criteria 01 can predict SCC development.

^b	B	Wald	Sig.	Exp (B)	PPV	NPV	Overall
Criteria 01	1.680	9.739	0.002	5.364	50.0%	84.3%	76.1%
Constant	-1.680	26.157	0.000	0.186			

^bdependent, SCC tumor; df, degree of freedom; criteria 01, chibby nucleus and cytoplasmic area <1% and β -catenin cytoplasmic intensity greater than or equal to intermediate, membrane intensity, strong; B, association index; Wald, regression weight; sig.=P-value; Exp (B), odds ratio; PPV, positive predicting value; NPV, negative predicting value; overall, accuracy.

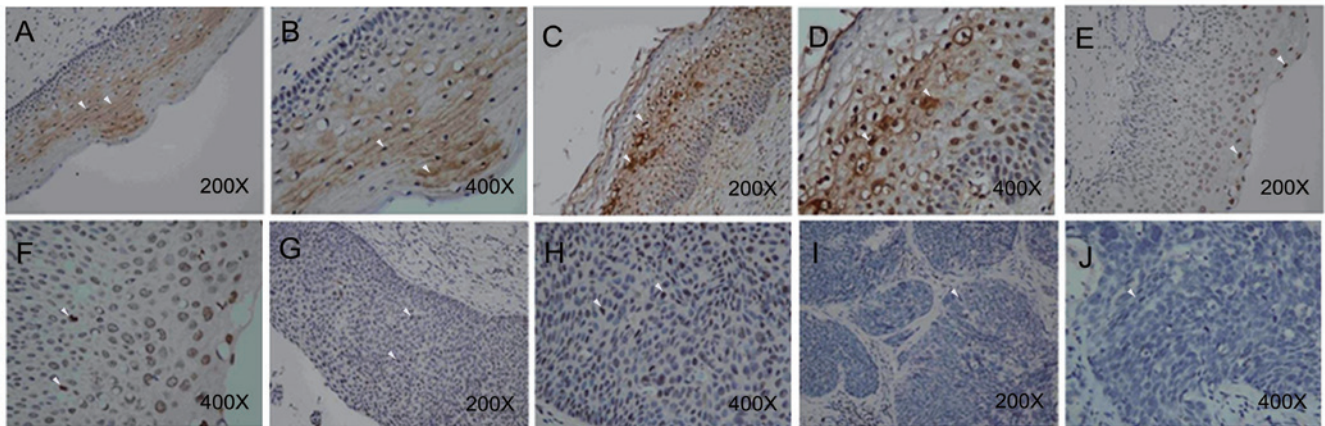


Figure 2. Expression of Chibby (an antagonist of β -catenin) in different CIN tumor stages. Immunohistological staining was performed using a NovoLink polymer detection kit. A Nikon Eclipse 50i light microscope was used at magnifications of x40-400. In chronic cervicitis tissues, Chibby was observed either in nucleus or in cytoplasm at (A) magnification, x200; and (B) magnification, x400. In CIN 1 tumor, Chibby was observed either in nucleus or in cytoplasm at (C) magnification, x200; and (D) magnification, x400. Chibby was only observed in the nucleus in partial CIN 2 tumors at (E) magnification, x200; and (F) magnification, x400. G. Nuclear Chibby expression was detected in CIN 3 tumors at (G) magnification, x200; and (H) at magnification, x400. The nucleus and cytoplasm lacked Chibby expression when observed at (I) magnification, x200; and (J) at magnification, x400. CIN, cervical intra-epithelial neoplasia. Left panel of images (A, C, E, G and I) were captured at x200 and right panel of images (B, D, F, H, and J) were captured at x400. White arrows indicate positive staining signal.

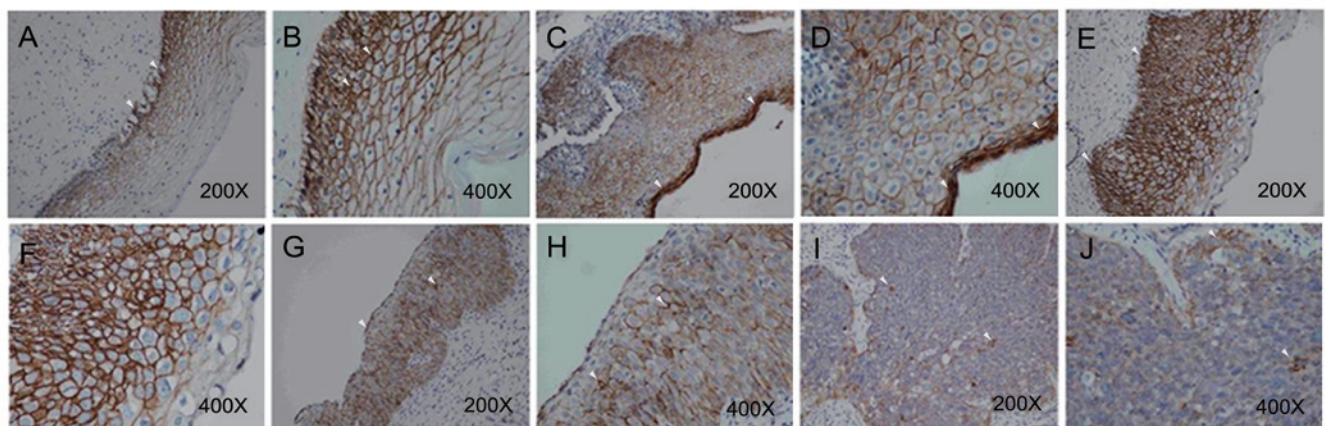


Figure 3. β -catenin expression in different CIN stage tumors. Immunohistological staining was performed using a NovoLink polymer detection kit. A Nikon Eclipse 50i light microscope was used at magnifications of x40-400. (A) Nuclear expression of β -catenin was not observed in normal cervix at magnification, x200; however, (B) β -catenin was observed in the cytoplasm, principally localized in sites adjacent to cell membrane at magnification, x400. (C) Nuclear expression of β -catenin was also not observed in CIN 1 tumors at magnification, x200; however, (D) β -catenin was detected in the cytoplasm at magnification, x400. β -catenin was observed, primarily in the cell membrane of CIN 2 tumors, at (E) magnification, x200; and (F) at magnification, x400. β -catenin was observed, principally in the cell membrane of CIN 3 tumors, at (G) magnification, x200; and (H) magnification, x400. Cytosolic β -catenin was observed, principally in the cell membrane of SCC tumors, at (I) magnification, x200; and (J) magnification, x400. White arrows indicate positive staining signal. CIN, cervical intra-epithelial neoplasia; SCC, squamous-cell carcinoma.

Table IV. Chibby nucleus (P=0.001) and cytoplasmic (P<0.001) positive staining area and β -catenin cytoplasmic (P=0.006) and membrane intensity (P=0.018) were independently associated with CIN stage, but not the Chibby cytoplasmic intensity

($P=0.062$). On the basis of logistical regression, the data was categorized according to the criteria 01, by which samples with a Chibby nucleus and cytoplasmic positive staining area $<1\%$, β -catenin cytoplasmic intensity was greater than or equal to intermediate, and membrane intensity was identified as strong staining were indicated as 1; whereas the counterparts were indicated as 0. The dummy variable of criteria 01 was brought to binary logistic regression to predict SCC. As Table V presents, criteria 01 was significantly associated with SCC ($P=0.002$) and samples that fulfilled criteria 01 exhibited a 5.364-fold increased risk of developing SCC. The PPV was 50.0%, the NPV was 84.3% and the accuracy was 76.1%. The results indicated that determining the protein expression of Chibby and β -catenin, on the basis of criteria 01, may serve as biomarker to predict invasive SCC.

Discussion

Abnormal activation of the Wnt signaling pathway is typically observed in a number of types of cancer cell (30). β -catenin stability may determine the activation of the Wnt signaling pathway. In quiescent cells, cytosolic β -catenin is decreased due to its degradation by the ubiquitin-proteasome dependent pathway; however, when β -catenin is translocated to the nucleus, it binds to LEF/TCF, and consequently activates basal transcriptional factor and a subset of downstream genes comprising cyclin D1, c-myc, c-jun, and matrix metalloproteinases 2 and 7. The activation of these genes may prompt cancer cell proliferation and have been associated with cancer cell invasiveness (31-33). In contrast to β -catenin protein expression and localization, β -catenin mRNA was less likely to be associated with tumorigenesis (34). Generally, the nuclear translocation of β -catenin is thought to be one of major causes of tumorigenesis (35). Furthermore, β -catenin is dysregulated and has been identified to accumulate in cancer cells; therefore, the abnormal accumulation observed in cervical cancer was not surprising (36-38). Increased expression of β -catenin may accelerate proliferation and differentiation in malignant cervical cancer, including CIN 3 tumors. In CIN 3 malignant tumors, increased proliferation and migration are indicators of the patient outcome. Previous studies have revealed that β -catenin expression was associated with patient survival (39-42). Decreased expression of β -catenin is associated with the long-term survival of patients with brainstem gliomas (43). In cervical adenocarcinomas, β -catenin expression has been associated with a poor 10-year survival, and detecting β -catenin is suggested as a prognostic marker of cervical adenocarcinoma (44).

In the normal cervix, we suggested that cytosolic and nuclear expression of β -catenin is hypothesized to be antagonized by Chibby, as previously a reported phenomenon in a mammalian cell line (45). Increased expression of β -catenin is hypothesized to be associated with highly proliferative and differentiating cancer cells, and expected to be observed in the majority of types of cancerous tissues (46). In the present study, β -catenin was highly expressed in advanced CIN stage tumors, in particular, in invasive SCC. Additionally, β -catenin gradually accumulated as the CIN stage increased, between CIN 2 and SCC. This result is consistent with current hypotheses about β -catenin expression in cervical cancer (47,48). Notably,

in the present study, β -catenin expression was restricted to the cytoplasm in all tissues. Furthermore, β -catenin principally accumulated near the cell membrane and lacked expression within the nucleus. The nuclear translocation of β -catenin was not detected in tumors of higher CIN stage, or even in CIN 1 lesions. This finding is in agreement with a previous study in which β -catenin was observed to be expressed in the cell membrane of pre-malignant tumors, but not cancerous tissues (38). In the present study, membrane β -catenin was identified to be more abundant in CIN 1 lesions compared with cytosolic β -catenin. In the present study, between 11 and 50% positive staining of cytosolic β -catenin and an intermediate level of membrane β -catenin was observed more frequently in pre-malignancy tissues and decreased in tumors of high CIN stages. Furthermore, β -catenin mRNA and protein were not differently expressed between CIN 2 and invasive SCC; however, intermediate-level cytosolic β -catenin accumulated between chronic cervicitis and invasive SCC. These results were consistent with that of a previous study, where β -catenin expression was not significantly different in SCC but was increased in high-grade squamous intraepithelial lesions (equal to CIN2-CIN3) and SCC, compared with pre-malignant lesions (14). Thus, discrepancies in β -catenin protein expression between pre-malignant and cancerous tissues in previous studies may be due to different definitions of 'positivity' in data interpretation. The results of the present study indicated that increased levels (intermediate to strong) of β -catenin protein accumulation may be associated with tumorigenesis in cervical cancer; however, its role in advanced disease progression remains unknown.

Chibby is an antagonist involved in the Wnt/ β -catenin signaling pathway and is hypothesized to serve a function in tumorigenesis. Previous studies have found that Chibby is downregulated in a number of types of cancer including neuroblastoma (19), lung cancer (20), ependymoma (21) and colorectal cancer (22,23). In neuroblastoma, Chibby has been suggested to interact with two tumor suppressors, neuroblastoma breakpoint family member 1 and clusterin (19). In a colon cancer line, Chibby was identified to bind to 14-3-3 protein and enabled β -catenin to be sequestered for nuclear exportation (23). Additionally, Chibby has been revealed to regulate β -catenin expression in lung cancer (20). In spite of these previous results, the associations between Chibby, pre-malignancy and tumorigenesis in cervical cancer remain unknown. Compared with chronic cervicitis tissue, nuclear and cytosolic Chibby expression was significantly decreased in lesions with a high CIN stage ($P<0.001$). For nuclear Chibby staining, the expression levels varied with CIN stage, with tumors of higher CIN stage progressively expressing decreased nuclear Chibby ($P<0.001$). For cytosolic Chibby staining, increased protein expression was determined in CIN 1 lesions compared with later CIN tumor stages. The results of the present study indicated that the downregulation of Chibby in the nucleus and cytoplasm is associated with tumorigenesis in cervical cancer. Furthermore, Chibby expression is negatively associated with disease progression, suggesting that Chibby is a functional tumor suppressor. In the present study, it was hypothesized that Chibby is involved in the Wnt signaling pathway and antagonizes the β -catenin interaction with TCF/LEF. In addition, Chibby

may coordinate with 14-3-3 to execute nuclear exportation of the TCF/LEF- β -catenin complex and downregulate downstream gene expression in the Wnt signaling pathway, as described previously (17,18). It may be hypothesized that when nuclear Chibby expression is decreased or absent, accumulation of the TCF/LEF- β -catenin complex, due to the relatively decreased Chibby/ β -catenin ratio, may subsequently trigger downstream Wnt signaling pathway gene expression, leading to cell proliferation and cervical cancer tumorigenesis.

Our previous study demonstrated that the Chibby mRNA and protein are downregulated in two cervical cancer lines, HeLa and SiH (Huang Y.L., unpublished work). In this study, a significant difference in Chibby mRNA expression and the Chibby/ β -catenin ratio between different CIN groups was determined. In addition, nuclear and cytosolic Chibby expression were significantly downregulated in high CIN stage tumors, in particular, when Chibby expression was interpreted by positive staining area (nucleus, >50% area; cytoplasm, 11-50% area). According to the results of the present study, Chibby nuclear and cytoplasmic positive staining area and β -catenin cytoplasmic and membrane intensity were associated with CIN staging. Therefore, the criteria 01 was constructed and converted into a dummy variable for logistical regression, to determine whether it may predict SCC. Criteria 01 was significantly associated with SCC ($P=0.002$), with PPV and NPV of 50.0 and 84.3%, respectively. In terms of clinical relevance, the negative prediction rate of a biomarker is relatively important since it may be beneficial to rule out false positive cases during the risk assessments. Furthermore, it is not affected by disease prevalence as PPV. The results of the present study indicated that determining the protein expression of Chibby and β -catenin, on the basis of criteria 01, may serve as biomarker for predicting invasive SCC of cervical cancer.

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